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(71) Applicant (for all designated States except US): **VALIO LTD** [FI/FI]; Meijeritie 6, FIN-00370 Helsinki (FI).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **MÄYRÄ-MÄKINEN, Annika** [FI/FI]; Maurinkatu 4 B 19, FIN-00170 Helsinki (FI). **SUOMALAINEN, Tarja** [FI/FI]; Valkealantie 10 A 1, FIN-00950 Helsinki (FI). **VAARALA, Outi** [FI/FI]; Fredrikinkatu 75 A 5, FIN-00100 Helsinki (FI).

(74) Agent: **KOLSTER OY AB**; Iso Roobertinkatu 23, P.O. Box 148, FIN-00121 Helsinki (FI).

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(54) Title: COMBINATION OF PROBIOTICS

(57) Abstract: The invention relates to a probiotic combination comprising different combinations of lactobacilli, propionic acid bacteria and/or bifidobacteria. The probiotics are preferably combined with a suitable prebiotic to form a synbiotic. The combination of the invention can be consumed as such or combined with a suitable foodstuff or pharmaceutical product, and it is therapeutically useful for example for stimulating the immune system and for general health improvement.

COMBINATION OF PROBIOTICS

FIELD OF THE INVENTION

[0001] The invention relates to a combination of probiotics, the combination comprising lactobacilli, propionic acid bacteria and/or bifidobacteria in various combinations. The probiotics are preferably combined with a suitable prebiotic to produce a synbiotic. The combination of the invention may be consumed as such or combined with a suitable foodstuff, such as a dairy product or a drink, and it is therapeutically useful for example for stimulating the immune system and for general health improvement.

10 BACKGROUND OF THE INVENTION

[0002] Probiotics are live microbes which, when administered to man or animals, promote the well being of the host by improving the intestinal microbial balance (Fuller, R. Probiotics in man and animals, 1989, J. Appl. Microbiol. 66:365-378). The best-documented probiotics include *L. rhamnosus* LGG, *L. johnsonii* LAI, *L. casei* Shirota and *Bifidobacterium lactis* Bbl2. In addition, a number of other probiotics have been described in the literature of the art (see for example M. E. Sanders & J. H. in't Veld 1999, Antonie van Leeuwenhoek 76:293-315, Kluwer Academic Publishers). The health-promoting effects of probiotics include the balancing and maintenance of intestinal flora, stimulation of the immune system and anti-carcinogenic activity. The useful effects of probiotics in human intestines are based on several factors caused by live bacterial cells, their cell structures and metabolic products. Probiotics are usually administered in nutrients or as capsules.

[0003] A bacterium may be referred to as a probiotic if it essentially meets the following requirements (Lee, Y-K and Salminen, S. 1995 The coming age of probiotics. Trend Food Sci Technol, 6:241-245): it remains viable in the demanding conditions prevailing in the digestive tract (low pH of the stomach, acids of the digestive system, etc.); attaches to the walls of the intestine; metabolizes in the intestine; is technologically applicable (endures processing); exhibits clinically tested and reported health effects; and is safe to consume.

[0004] Prebiotics are nondigestible food ingredients which promote the health of humans by selectively stimulating the growth and activity of one or some probiotic bacteria in the colon (Gibson, G.R. and Roberfroid, M.B. 1995. Dietary modulation of the human colonic microbiota - introducing a concept of prebiotics. J. Nutr. 125:1401-1412). A prebiotic is usually a

nondigestible carbohydrate (oligo- or polysaccharide) or a sugar alcohol which is not degraded or absorbed in the upper digestive tract. Known prebiotics used in commercial products include inulin (fructo-oligosaccharide, or FOS) and transgalacto-oligosaccharides (GOS or TOS).

- 5 **[0005]** A synbiotic is defined as a combination of a prebiotic and a probiotic, the prebiotic promoting the viability of the added microbe and its attachment to the intestine, thereby promoting health (Gibson and Roberfroid 1995, supra). When nondigestible carbohydrates that have passed through the small intestine are fermented in the colon, short-chain fatty acids, other
10 organic acids, alcohols, hydrogen and carbon dioxide, for example, are formed (Gibson and Roberfroid 1995, supra). The primary fatty acids produced in fermentation are acetic acid, butyric acid and propionic acid (Cummings, J.H. Short-chain fatty acids, in: Human Colonic Bacteria: Role in Nutrition, Physiology and Pathology, G.R. Gibson and G.T. Macfarlane (eds.), pp. 101 -
15 130, CRC Press, Boca Raton, 1995). An increase in the number of short-chain fatty acids would be generally advantageous. Nondigestible carbohydrates are the principal substrate for colonic microbes, although they also may include compounds the intestinal fermentation of which is disadvantageous (Gibson and Roberfroid, 1995, supra).
- 20 **[0006]** Human digestive tract accommodates a plural number of bacteria which live in symbiosis with the host. There are great differences in microbial content between the different parts of the tract, about 95% of all the intestinal bacteria appearing in the colon, which is the most important part of the intestines. Over 400 bacterial species have been estimated to thrive in the
25 colon. In addition to these, the intestines contain microbes known as transient microbes (G.R. Gibson and M.B. Roberfroid (eds.) Colonic Microbiota; Nutrition and Health. Kluwer Academic Publisher, Dordrecht, 1999). The dominating species are the following: *Bacteroides*, *Bifidobacterium*, *Coprococcus*, *Peptostreptococcus*, *Eubacterium* and *Ruminococcus*. The number of species *Lactobacillus*, *Streptococcus*, *Fusobacterium*, *Veillonella*,
30 *Propionibacterium* and *Enterobacteriaceae* is slightly less. Some of the species represent useful microbes, whereas others may even be harmful. The average microbial content in feces is 10^{12} cfu/g (per dry matter). Bacteria degrade and ferment those food ingredients in the colon, which are not absorbed in the small
35 intestine, the end products of the fermentation being absorbed in the intestine for use by the body. In addition to nutrition, the microbial balance of the colon is

of major significance to the state of health of a man (Tannock, G.W. 1998. Studies of the intestinal microflora: A prerequisite for the development of probiotics, Int. Dairy J. 8:527 - 533). Changes in the composition of the intestinal flora or a sudden reduction in the amount of it (due to severe diarrhea, antibiotics treatment, etc.) increase the infectivity of potentially pathogenic species, which may have serious consequences (outbreak of allergies, intestinal diseases, cancer).

[0007] The β -glucuronidase enzymes produced by intestinal bacteria are assumed to contribute to the formation of carcinogenic compounds, for example. Steroids and other carcinogenic compounds metabolize in the liver and then conjugate with glucuronic acid. The bile delivers the conjugated glucurone compound to the small intestine and from there the compound passes further to the colon where the glucuronidase enzymes can hydrolyse the compound, thereby releasing toxic compounds into the colon (Rowland, I.R. 1995. Toxicology of the colon: role of the intestinal microflora, in: Human Colonic Bacteria, Role in nutrition, physiology, and pathology. Editors: Gibson, G.R. and Macfarlane, G.T., pp. 155-174, Boca Raton: CRC Press). It is assumed that species of *Eubacterium*, *Bacteroides* and *Clostridium* release greater amounts of these harmful enzymes into the intestines than representatives of species of *Bifidobacterium* and *Lactobacillus*. This would thus provide one reason why it would be advantageous that the intestinal flora is composed of bifidobacteria and lactobacilli.

[0008] In addition, glycosides originating from vegetables and tea, for example, are not absorbed in the small intestine and pass to the colon, where they may be hydrolysed by the action of β -glucosidases to form toxic or mutagenic aglycone compounds (Goldin, B.R. 1990. Intestinal Microflora: metabolism of drugs and carcinogens. Annals of Medicine 22:43-48).

[0009] Moreover, the intestinal flora produces urease enzyme which degrades urea into ammonia. High amounts of ammonia may be toxic to the epithel cells of the intestine (Mobley, H.L.T. and Hausinger, R.P. 1989. Microbial ureases: significance, regulation and molecular characterization. Microbiological Reviews 53:85-108).

[0010] Human intestinal flora is formed during the early years of life and no major changes take place in its composition thereafter. Only minor changes within the species may take place (in bifidobacteria, for example).

[0011] Along with the increased understanding of the importance of intestinal flora, research has thus been actively focused on discovering the factors that can be used to influence the composition of the flora and its operation (viability) in such a way that beneficial bacterial species would be strengthened and harmful ones reduced. It is assumed that the operation of microbes can be influenced by prebiotics that promote beneficial bacteria. Extensive research has been carried out into galactooligosaccharides (GOS) which are di-, tri-, tetra-, penta- and hexasaccharides and which primarily contain galactose units. They are prepared enzymatically from lactose and the content of the end product depends on the enzyme used (Matsumoto, K. et al. 1993. Galactooligosaccharides, in: Oligosaccharides. Production, properties and applications. Ed. Nakakuki, T., Japanese Technology Reviews. Vol. 3. No. 2., pp. 90-116, Gordon and Breach Science Publishers, Switzerland, Australia). GOS has earlier been demonstrated to exhibit for example bifidogenic properties, i.e. those promoting the growth of bifidobacteria (Ito, M. et al. 1990. Effect of administration of galactooligosaccharides on the human faecal microflora, stool weight, and abdominal sensation. Microb. Ecol Health Dis. 3:285-292).

DESCRIPTION OF THE BACKGROUND ART

[0012] Both products containing an individual probiotic strain and combinations of a plural number of different probiotics have been abundantly described in the literature of the art. Synbiotics have also been described in the literature of the art.

[0013] Publication EP 904 784, N. V. Nutricia, for example, describes a probiotic product containing *Bifidobacterium*, *Enterococcus faecium* and *Lactobacillus*. In addition, the product may contain prebiotics, such as polysaccharide or non-degradable starch, and immunoglobulines, vitamins, etc. According to the publication, the product has a health-promoting effect in that it stimulates the immune system, for example. The effect has not, however, been shown in clinical tests, neither has any other biological activity been studied.

[0014] WO 00/33854, N. V. Nutricia, describes a product comprising a probiotic and oligosaccharides. The probiotics particularly referred to are *Lactobacillus* and *Bifidobacterium*, although *Pediococcus*, *Propionibacterium*, *Leuconostoc* and *Saccharomyces* are also mentioned. The

prebiotics involved include transgalactooligosaccharides (TOS) and fructooligosaccharides (FOS). According to the publication, a particularly advantageous combination comprises *Lactobacillus rhamnosus* and a transgalactooligosaccharide or a hydrolysis product of potato galactane, this combination and its preparation being also illustrated in the examples. According to the publication, the product has health-promoting effects and it is particularly useful in the treatment of intestinal disorders. However, its activity has not been demonstrated in any way.

[0015] WO 97/34615, University of New South Wales, describes a probiotic composition containing, in addition to one or more probiotics, resistant (non-degradable) starch and oligosaccharide, a synergistic effect being obtained between the three components. The probiotics mentioned are *Saccharomyces*, *Bifidobacterium*, *Bacteroides*, *Clostridium*, *Fusobacterium*, *Propionibacterium*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Staphylococcus*, *Peptostreptococcus* and *Lactobacillus*, and the oligosaccharides include, among many others, fructo- and galacto-oligosaccharides. The examples show the synergistic effect of bifidobacteria, maize starch and fructooligosaccharides on the amount of bifidobacteria. Any therapeutic effect, however, has not been described.

[0016] US 5,895,648, Sitia-Yomo S.p.A., describes a probiotic composition comprising, in the form of lyophilized, live bacteria, at least two species of bifidobacteria and at least two species of lactobacilli or streptococcus combined with one or more oligosaccharides. The composition comprises altogether 4 to 20 parts by weight of probiotics and 5 to 22 parts by weight of oligosaccharides, of which for example galacto- and fructooligosaccharides, inuline in particular, are mentioned. The probiotics mentioned include *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Streptococcus thermophilus* and *Streptococcus faecium*. According to the publication, a mixture containing probiotics and prebiotics can be added to milk-based desserts, milks or juices to balance the functioning of the intestine. Any biological activity of the combination has not, however, been described.

[0017] The publication *Milchwissenschaft* (1988) Vol. 53, No. 11, pp. 603-605, describes PAB-milk which comprises three bacterial strains: *Propionibacterium freudenreichii* subsp. *shermanii* MTCC 1371, *Lactobacillus*

acidophilus R. and *Bifidobacterium bifidum* NDRI. Prebiotics have not been added to the product. According to the publication, PAB-milk is suitable for babies and children, also for those suffering from lactose intolerance.

5 **[0018]** WO 99/10476 describes the stimulating effect of specific bacterial strains, i.e. *Lactobacillus rhamnosus* HN001 (NM97/09514) and HN067 (NM97/01925), and *Lactobacillus acidophilus* HN017 (NM97/09515) and *Bifidobacterium lactis* HN019 (NM97/09513), on the immune system, measured as enhanced phagocytosis. The strains can be used individually, or added to dairy products or pharmaceutical preparations.

10 **[0019]** US 5,902,578, Abbott Laboratories, relates to a method for preventing and treating diarrhea with a mixture of *Lactobacillus reuteri*, *Lactobacillus acidophilus* and *Bifidobacterium infantis*, the mixture being prepared as a powder, liquid or pills.

15 **[0020]** Biological and therapeutic effects of probiotics and synbiotics of the above type have also been described in the background art. As examples may be mentioned Gallagher, D. et al. Journal of Nutrition (1996) Vol. 126, No. 5, pp. 1362-1371, which describes the effects of bifidobacteria and *Lactobacillus acidophilus* on colon cancer in rats and states that the best results were obtained by using both the bacteria and a fructooligosaccharide, and Kirjavainen, P. et al. Clinical and Diagnostic Laboratory Immunology (Nov 20 1999) Vol. 6, No. 6, pp. 799-802, which describes the positive effects of two, separately studied, lactic acid strains, *Lactobacillus rhamnosus* GG and *Propionibacterium freudenreichii* subsp. *shermanii* JS, on the lymphocyte levels, and thereby the immune response, of mice. The combination of these 25 two bacteria has not been described or studied.

30 **[0021]** The combining of probiotics with other substances having therapeutic effects has also been described in the literature of the art. For example, WO 97/29762 and WO 97/29763, Procter & Gamble Company, describes the use of lactobacilli and bifidobacteria combined with galacto- or fructooligosaccharides together with a plant of the genus *Ericaceae*, or an extract thereof for the treatment of urinary tract infections and intestinal disorders, and WO 00/29007, Reddy, describes the combining of probiotics, such as *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Propionibacterium*, *Brevibacterium*, *Penicillium* and *Saccharomyces* with herb- 35 based natural products and drug-like substances.

[0022] Although probiotics and synbiotics have been extensively studied, good and versatile commercial products are not available to any significant extent. Consequently, there is continued, evident need to offer the consumers new products having clearly demonstrated probiotic effects and
5 produced in a form that allows them to be used as a convenient part or supplement, for example, of the every-day diet.

BRIEF DESCRIPTION OF THE INVENTION

[0023] It is therefore an object of the present invention to provide a new probiotic-containing product the probiotic effect of which has been clearly
10 demonstrated, which is pleasant to use, and healthy for the consumer.

[0024] These objects were achieved with a new combination of the invention which comprises a plural number of probiotics. The present invention is thus based on a new combination comprising (2) lactobacilli strains, a propionic acid bacterium and/or a bifidobacterium. In addition, the combination
15 preferably comprises a prebiotic supporting the growth of the above-mentioned microbes.

[0025] According to the invention, two strains of lactobacilli are mainly used, i.e. *Lactobacillus rhamnosus* LGG (ATCC 53103) and *Lactobacillus rhamnosus* LC705 (DSM 7061). The propionic acid bacterium is
20 usually *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM 7067). The bifidobacterium may be any bifidobacterium having a probiotic effect, typically strains belonging to the species *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium bifidum* and *Bifidobacterium adolescentis* are used.

[0026] At least three of these bacteria are usually included, and the combination preferably includes *Lactobacillus rhamnosus* LGG and/or *Propionibacterium freudenreichii* ssp. *shermanii* PJS.
25

[0027] The most preferred combination is a mixture of four strains, *Lactobacillus rhamnosus* LGG (ATCC 53103), *Lactobacillus rhamnosus* LC705 (DSM 7061), *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM 7067)
30 and *Bifidobacterium infantis* Bbi99 (DSM 13692). However, any bifidobacterium (such as Bbl2) may be included in the combination, if desired. The prebiotic used is preferably galactooligosaccharide (GOS).

[0028] Another preferred composition is a combination of a
35 bifidobacterium and a propionic acid bacterium, in which any bifidobacterium

can be used together with *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM 7067).

[0029] The new combination can be used as such or as a part of another product, such as a pharmaceutical or a food product. The combination
5 of the invention has an advantageous effect on the human intestinal balance in that it increases the entero-lactone production and reduces a disadvantageously high pH value. The combination also influences the immune response by increasing the amount of lymphocytes and that of γ -interferon (IFN) and by reducing the formation of carcinogenic substances. The combination of the
10 invention is thus useful for the prevention and treatment of intestinal disorders, allergies and cancer and for promoting general health.

[0030] In accordance with the present invention, the combination is thus also applicable as a therapeutic substance and in the preparation of therapeutic substances.

15 DETAILED DESCRIPTION OF THE INVENTION

[0031] Of the strains used in the invention, *Lactobacillus rhamnosus* GG (LGG), *Lactobacillus casei* ssp. *rhamnosus* LC705 and *Propionibacterium freudenreichii* ssp. *shermanii* JS (PJS) have been described in the prior art. *Bifidobacterium infantis* Bbi99, which may be included in the combination, is a
20 new strain and will be described in greater detail below.

[0032] *Lactobacillus rhamnosus* GG (LGG) is described for example in US Patent 5,032,399, Gorbach & Goldin. The strain is isolated from human feces, it is able to grow well in pH 3 and survives even lower pH values as well as high bile acid contents. The strain exhibits excellent adhesion to both mucus
25 and epithelial cells. Lactic acid yield from glucose is good: when grown in MRS broth, the strain produces 1.5 - 2% of lactic acid. The strain does not ferment lactose and thus it does not produce lactic acid from lactose. The strain employs the following carbohydrates: D-arabinose, ribose, galactose, D-glucose, D-fructose, D-mannose, rhamnose, dulcitol, inositol, mannitol, sorbitol, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose,
30 saccharose, trehalose, melezitose, gentibiose, D-tagatose, L-fucose, and gluconate. The strain grows well at +15 - 45°C, the optimum temperature being 30 - 37°C. *Lactobacillus rhamnosus* GG is deposited with the depository authority American Type Culture Collection under accession number ATCC
35 53103.

[0033] *Lactobacillus casei* ssp. *rhamnosus* LC705 is described in greater detail in FI Patent 92498, Valio Oy. LC705 is a gram-positive short rod occurring in chains; it is homofermentative; weakly proteolytic; grows well at +15 - 45°C; does not produce ammonia from arginine; is catalase-negative; when
5 grown in MRS broth (LAB M), the strain produces 1.6% lactic acid having an optical activity of the L(+) configuration; the strain decomposes citrate (0.169%), thereby producing diacetyl and acetoin; the strain ferments at least the following carbohydrates (sugars, sugar alcohols): ribose, galactose, D-glucose, D-fructose, D-mannose, L-sorbose, rhamnose, mannitol, sorbitol,
10 methyl-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, melezitose, gentiobiose, D-turanose and D-tagatose. LC705 adheres weakly to mucus cells, but moderately to epithelial cells. The viability of the strain is good in low pH values and high bile acid contents. The strain survives well a salinity of 5% and fairly well a salinity of 10%. *Lactobacillus casei* ssp. *rhamnosus* LC705 is deposited
15 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) under accession number DSM 7061.

[0034] *Propionibacterium freudenreichii* ssp. *shermanii* JS (PJS) is also described in greater detail in FI Patent 92498, Valio Oy. PJS is a gram-
20 positive short rod; it ferments glucose, fructose, galactose and lactose; it ferments well lactate; and its optimum growth temperature is 32°C. The viability of the strain in low pH values and high bile acid contents is excellent. *Propionibacterium freudenreichii* ssp. *shermanii* JS is deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)
25 under accession number DSM 7067.

[0035] *Bifidobacterium infantis* Bbi99 has been isolated from the feces of a healthy newborn. *B. infantis* Bbi99 is a gram-positive pleomorphic rod. The strain is catalase-negative, fructose-6-phosphate-phosphoketolase-positive (F6PPK) and both α - and β -galactosidase- and α - and β -glucosidase-positive. *B.*
30 *infantis* Bbi ferments the following carbohydrates: ribose, galactose, D-glucose, D-fructose, D-mannose, methyl-D-mannose, N-acetylglucosamine, esculin, salicin, cellobiose, maltose, lactose, melibiose, and gentibiose. The optimum growth temperature is 30 - 40°C and pH 6.5 - 7.0. When grown in broths comprising hexose, the strain produces L-lactic acid and acetic acid (in the ratio of 2:3). The G+C content of DNA is 55 - 67 mol%. *Bifidobacterium infantis*
35 Bbi99 was deposited at the Deutsche Sammlung von Mikroorganismen und

Zellkulturen GmbH (DSM), in accordance with the Budapest Treaty, under accession number DSM 13692 on 28 August 2000.

[0036] The combination of the invention may also include other microorganisms, such as microorganisms and probiotics contained in starters
5 used in the dairy industry. There are numeral well-documented strains of starters, which are commercially available from producers such as Hansen A/S, Denmark, and Danisco/Wiesby GmbH, Germany.

[0037] To prepare the combinations of the invention, the microorganisms are cultivated using processes conventional in the art. They
10 can be cultivated as pure cultures or as different mixed cultures. The cultures can be used as such or they may be processed as desired for example by purifying, concentrating, lyophilising or finishing them to produce various preparations.

[0038] A sufficient amount of probiotics is used in the combination
15 to produce the desired probiotic effect. The amount of each probiotic may thus vary within a broad range, depending on, for example, the strains used and their number, the total amount of cells of the probiotics, the total daily dose, and on other properties and ingredients of the product. A daily dose of the combination usually comprises about 10^6 - 10^{10} cfu of probiotics.

[0039] One or more prebiotics are preferably added to the
20 combination to form a synbiotic. The prebiotic is selected according to the microorganisms included in the combination concerned such that it will support the growth of the microorganisms. Suitable prebiotics may include e.g. oligosaccharides, particularly galactooligosaccharide (GOS),
25 palatinoseoligosaccharide, soybean oligosaccharide, gentiooligosaccharide, xylooligomers, non-degradable starch, lactosaccharose, lactulose, lactitol, maltitol, polydextrose, or the like. A sufficient amount of the prebiotic is added to the synbiotic to produce a prebiotic effect. What is a sufficient amount is determined for example according to the strain concerned, the amount of the
30 prebiotics included and the other contents and the application of the product. The amount therefore also varies within a wide range; it may be from 0.5 to 5 g in a daily dose, for example.

[0040] The prebiotic does not necessarily need to be included in the combination. Depending on the end product and the purpose of use, it may be
35 better to consume the prebiotic separately, although approximately at the same time with the probiotic combination. In some cases it may suffice to only

consume the probiotic combination, the prebiotic being thus not needed at all. An example of this is a case where the intestinal conditions of the host are suitable for the growth of probiotics without an added prebiotic being needed, and when the prebiotic is contained in the normal diet (if it is consumed in porridge or rye bread, for example).

[0041] The present invention has shown that the microorganisms used meet the criteria set for probiotics: they survive well in the demanding conditions of the digestive tract, adhere well to intestinal cells and multiply well in the intestines. They have also been shown to exhibit excellent biological effects; for example, they increase the number of microorganisms desirable from the point of view of health and reduce the number of harmful microorganisms in the intestine, they reduce the activity of harmful enzymes and thereby the formation of harmful, or even carcinogenic, substances, and they have a stimulating effect on the immune response.

[0042] The combination of the invention can be used as such or in the form of capsules, pills or tablets, for example, manufactured in conventional processes of preparing pharmaceutical products. The combination of the invention may also be added to diverse edible products, such as foodstuffs, products of the beverage or confectionery industry, health-promoting products, natural products, etc. In the context of the present invention, products containing the combination of the invention, such as dairy products, particularly yogurts and other fermented milk products; cheeses and spreads; children's food; juices and soups; and capsules are preferred. A product in the form of a capsule usually only contains the probiotic combination, the prebiotic being consumed separately.

[0043] The end products are prepared in conventional processes, the combination being added either in connection with the preparation or thereafter, during the finishing of the end product.

[0044] The invention is described in greater detail with reference to the following examples, which are only intended to illustrate the invention and not to restrict its scope in any way.

Example 1

Preparation of the combination

[0045] The combination was prepared from a bacterial mixture, adding, when desired, galacto-oligosaccharide (GOS) as a prebiotic. The bacterial mixture was formed from bacterial cultures (concentrates or freeze-

dried powders) of four strains, i.e. *Lactobacillus rhamnosus* LGG (ATCC 53103), *Lactobacillus rhamnosus* LC705 (DSM 7061), *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM 7067) and *Bifidobacterium infantis* Bbi99 (DSM 13692).

5 **[0046]** Both LGG and bifidobacterium were cultivated individually.

[0047] LGG was cultivated in a medium comprising 5.0% of whey permeate (Valio Oy), 0.5% of casein hydrolysate (Valio Oy), 0.5% of technical yeast, and 0.0015% of $\text{MnSO}_4 \times \text{H}_2\text{O}$. The components of the medium were dissolved into water and the medium was sterilized (for 20 min at 120°C). The cultivation was carried out at a temperature of 37°C and a pH of 5.8 (adjusted with NH_4OH) for about 18h and at a mixing rate of 100 rpm. After the cultivation the bacterial cells were concentrated, washed and freeze-dried using a 10% (v/v) protective agent supplement, such as a saccharose broth of 46%, or a similar alternative known to those skilled in the art. The final bacterial content was $>1 \times 10^9$ cfu/ml in the cultivation, $>1 \times 10^{10}$ cfu/g in the concentrate and $>1 \times 10^{11}$ cfu/g in the freeze-dried powder.

[0048] The composition of the growth medium of bifidobacteria was the following: whey permeate 4% (Valio Ltd.), casein hydrolysate 1.0% (Valio Ltd.), technical yeast extract 1.0% (LAB M), cystein-HCl 0.03% (Merck, Darmstadt, Germany). Other ingredients of the medium were first dissolved into water, then the cystein-HCl was added and the medium was sterilized (for 20 min at 120°C). The cultivation was carried out in about 18-20 hours at a temperature of 37°C and a pH of 6.7 (adjusted with NH_4OH) and at a mixing rate of 100 rpm. The bacterial content of the cultivation was $>1 \times 10^9$ cfu/ml. After the cultivation the bacteria cells were concentrated, washed and freeze-dried using a 10% (v/v) protective agent supplement, such as a saccharose broth of 46%, or a similar alternative known to those skilled in the art. The bacterial content in the concentrate was $>1 \times 10^{10}$ cfu/g and in the freeze-dried powder $>1 \times 10^{11}$ cfu/g.

[0049] LC705 and PJS were cultivated together by inoculating bacterial cells to a whey-based growth broth in a ratio of 1:2. The whey-based growth medium contained 3.5 - 5% of whey permeate (Valio Oy), 1.0% of caseinhydrolysate and 1.0% of yeast extract (Valio Oy). The strains were cultivated for three days at 30°C with the pH maintained at 4.5 by means of an automated pH adjustment. After the cultivation, the content of each bacterial strain was $>1 \times 10^9$ cfu/ml. After the cultivation the bacterial cells were concentrated, washed and freeze-dried using a 10% (v/v) protective agent

supplement, such as a saccharose broth of 46% or a similar alternative known to a person skilled in the art. The content of each of the bacteria in the concentrate was $>1 \times 10^{10}$ cfu/g and in the freeze-dried powder $> 1 \times 10$ cfu/g.

[0050] The strains may also be cultivated separately. In that case
5 LC 705 is cultivated as described above in connection with the joint cultivation, only the cultivation time is 1 day at 30°C. PJS is cultivated in a whey-based growth medium comprising 2% of whey permentate, 1.0% of casein-hydrolysate and 1% of yeast extract. The strain is cultivated for 3 days at 30°C in a pH value of 6.3, after which the process is carried out as above.

10 [0051] The concentrates or the powders are mixed in a ratio of 1:1:1. When LC705 and PJS have been cultivated separately, the mixing ratio is 1:1:1:1. The obtained mixture of concentrates or freeze-dried powders is used as a probiotic portion in the different product applications of the combination. The mixture is added to the product application to obtain the
15 following final bacteria content in the product:

	LGG	$>10^6$ cfu/g of the product
	LC705	$>10^6$ cfu/g of the product
	PJS	$>10^6$ cfu/g of the product
20	Bifidobacterium	$>10^6$ cfu/g of the product

[0052] GOS (Valio Oy) was added, when desired, as a separate product to the product application to obtain a GOS concentration of about 0.5 - 5 g/dose in the product.

25 **Example 2**

Adhesive properties of the strains and their tolerance in intestinal conditions

[0053] Adhesion of the probiotic strains to mucus was tested in accordance with Ouweland et al. (Ouweland, A. C., Kirjavainen, P. V.,
30 Grönlund, M. - M., Isolauri, E., and Salminen, S. J. 1999. Adhesion of probiotic micro-organisms to intestinal mucus. Int. Dairy J. 9:623-630). LGG and probiotic bacterium PJS adhered excellently to the intestinal mucus, Bbi99 moderately and LC705 weakly. Adhesion is a prerequisite for the microbe to produce beneficial effects in the intestines. On the other hand, LC705 is known to adhere
35 well to epithel cells, similarly as LGG (Lehto, E. and Salminen S. 1997. Adhesion of two *lactobacillus* strains, one *lactococcus* strain and one

propionibacterium strain to cultured human intestinal CaCO-2 cell lines. Bioscience Microflora 16: 13 - 17). This property is beneficial when there is an imbalance in the intestine and the protective mucus has been weakened.

5

Table 1. Adhesive properties of probiotic strains

Strain	Adhesion (+Std.)	%
LGG	26.3 ± 1.3	
LC705	0.7 ± 0.2	
PJS	24.9 ± 2.2	
Bbi99	4.6 ± 2.3	

[0054] *In vitro* tests have shown the strains to endure physiological bile salt contents and the low pH of the stomach.

- [0055]** The strains were tested at different pH values in MRS broths the pH of which had been adjusted with lactic acid to values pH 4, pH 3 and pH 2. The strain to be studied (fresh culture) was inoculated into a pH broth of 1% and grown at 37°C for 3 hours, after which the content of live cells was determined using an agar suitable for the strain (see Table 5). The strains maintained their viability in the 3-hour process in a pH value of 3 excellently.
- The propionic bacterium remained viable even in pH 2. In the test the bacteria were not protected by components carried by food (such as fat) and therefore they may be assumed to preserve even better when consumed *in vivo* together with food.

Table 2. Cell contents of strains cultivated in pH-adjusted MRS broths

	Initial content cfu/ml	pH 4 cfu/ml	pH 3 cfu/ml	pH 2 cfu/ml
Bbi99	1 × 10 ⁷	2 × 10 ⁷	<10	<10
PJS	1 × 10 ⁸	9 × 10 ⁷	3 × 10 ⁷	1 × 10 ⁴
LGG	1 × 10 ⁷	2 × 10 ⁷	1 × 10 ⁷	<10 ²
LC705	6 × 10 ⁷	2 × 10 ⁸	4 × 10 ⁷	<10 ²

[0056] With regard to bile salt tolerance, the strains were tested in MRS broths that contained 0.3% and 0.5% of Oxgal (Sigma) bile salt by inoculating 1% of the fresh culture into the bile salt MRS broth to be studied.

The strains were cultivated in the broths for 3 hours at 37°C, after which the content of live cells was determined using an agar suitable for the strain (see Table 5). All strains survived the treatment excellently.

5 Table 3. Cell contents of strains cultivated in bile salt contents of 0.3 % and 0.5 %

	Initial content (cfu/ml)	Bile salt 0.3 %	Bile salt 0.5 %
Bbi99	3×10^7	1×10^7	1×10^7
PJS	6×10^7	6×10^7	5×10^7
LGG	1×10^7	2×10^7	9×10^6
LC705	1×10^7	2×10^7	1×10^7

Example 3

Selecting a prebiotic suitable for the combination

10

[0057] Alternative prebiotics were studied on individual strains by cultivating each strain in a sugar-free MRS broth to which was added 1% of the prebiotic to be studied. Each strain was cultivated for 1-2 days at its optimum temperature. Bacterial growth was observed during the test by determining the turbidity of the cultivation by spectrophotometry. As is shown in Table 4, best promoter for growth in all the four strains was a galacto-

15

Table 4. The effect of prebiotics on bacterial growth

Prebiotic	LGG	LC705	PJS	Bbi99
GOS	++	++	++	+
FOS	-	-	-	-
Xylooligomers	+	++	+	+
Polydextrose	++	++	+	-
Arabinooligomers	-	-	-	-
Pectinooligomers	-	-	-	-
Xylitol	-	-	-	-
Maltitol	-	++	-	-
Lactitol	-	++	-	-

20

Example 4

Preparation of the end product

[0058] A functional drink (in Finnish 'tehojuoma'; Valio Oy) was used as a base for preparing a juice (dosage 65 ml/day) to which was added 0.1 g of freeze-dried mixture of bacteria / dose (= 65 ml) and 3.8 g of 70% GOS syrup / dose (= 65 ml). Corresponding juice with no added GOS syrup or bacterial mixture was used as a control.

[0059] The bacterial contents of the finished juice were as follows:

LGG	> 10 ⁷ cfu/ml
LC705	> 10 ⁷ cfu/ml
PJS	> 10 ⁸ cfu/ml
Bbi99	> 10 ⁷ cfu/ml

[0060] The product was used in the following clinical tests in which pro = juice + probiotic supplement and syn = juice + probiotic + prebiotic supplement.

Example 5

Clinical effects of the combination of the invention

[0061] The drink described in example 4, which contained the above described probiotic combination (Pro) or the probiotic combination and a prebiotic (Syn), was clinically tested on 20 males. The test persons took the drink daily in accordance with the study plan and during the study they were not allowed to consume any other probiotic-containing product. The study scheme was such that the test started with a run-in period continued by a probiotics period of two weeks and a subsequent synbiotics period, and it ended with what is known as a wash-out period.

The study scheme and schedule

week 8	N (week 11)	N (week 13)	N (week 15)	N (week17)
I 3 week	*I 2 week	*I 2 week	*I 2 week	*I
run-in	pro	Syn	normal diet	
without			without	
probiotic			probiotic	

[0062] At the end of each period, the test persons gave a feces sample and a blood sample (= N).

[0063] Microbes and enzymes were analysed from the feces samples and enterolactone content and immune response from the blood.

5.1. Microbes

Amount of microbes

- 5 [0064] The total lactic acid bacterial content, LGG, LC705, the total propionic acid bacterial content, PJS, and the total bifidobacteria content were determined applying methods known in the art and the parameters shown in Table 5.

10 **Table 5. Methods for determining microbes**

Determination	Agar	Cultivation temperature/ time	
Lactobacilli	MRS	37°C/3 days	anaerobic
LGG	MRS + 0.005 % vancomycin (Sigma)	37°C/3 days	
LC705	MRS + 0.005 % Vancomycin	37°C/3 days	
Propionic bacteria	Mod. YEL	30°C/7 days	anaerobic
PJS	Mod. YEL	30°C/7 days	anaerobic
Bifidobacteria	Raffinoseagar (RB)	37°C/2 days	anaerobic

Effect on the intestinal bacterial cell content *in vivo*

- 15 [0065] The contents of LGG, LC705 and PJS increased significantly in the samples of the test persons during the period they used the probiotic-containing product (Table 6). Since bifidobacteria contents were high from the very beginning of the test, changes have presumably only taken place within the species.

Table 6. Bacterial contents (log, cfu/g of feces; \pm std.)

	Initial content cfu/g	After intake of probiotic, cfu/g	After intake of synbiotic, cfu/g	Wash-Out cfu/g
MHB (tot)	6.0 ($\pm 1,2$)	6.1 ($\pm 1,2$)	6.1 ($\pm 1,0$)	4.5 ($\pm 1,6$)
LGG	2.3 ($\pm 1,0$)	4.7 ($\pm 1,7$)	5.3 ($\pm 1,2$)	3.0 ($\pm 1,6$)
LC705	2.0 (± 0)	5.2 ($\pm 1,3$)	5.4 ($\pm 1,0$)	2.8 ($\pm 1,1$)
Propion	2.7 ($\pm 1,4$)	5.7 ($\pm 1,6$)	5.6 ($\pm 1,4$)	2.4 ($\pm 1,7$)
Bifidobacteria	8.2 ($\pm 1,5$)	8.6 ($\pm 2,1$)	8.8 ($\pm 2,2$)	8.3 ($\pm 2,1$)

5 **[0066]** Addition of synbiotic to the product consumed in the intake test improved the viability of the added probiotics in the intestine. This is shown by the increase in the LGG content during the synbiotics period, for example.

Effect on pH value

10 **[0067]** The pH level of persons having an initial pH level of over 7 decreased in the groups that took the probiotic mixture and the synbiotic, whereas no decrease was observed in persons with an initial pH value lower than 7 (Table 7).

Table 7.

Change in pH after probiotic and synbiotic supplement

	Control	Probiotic alone	Synbiotic
pH>7	7.2	6.9	6.7
pH<7	6.6	6.7	6.6

5.2. Enzymes

20 **[0068]** The feces samples were processed as described by Ling et al. (Ling, W-H., Korpela, R., Mykkänen, H., Salminen, S., and Hänninen, O. 1994 Lactobacillus GG supplementation decreases colonic hydrolytic and reductive activities in healthy female adults. Journal of Nutrition 124, 18-24).

25 **[0069]** β -glucuronidase and β -glucosidase were determined as described by Freeman (Freeman, H. J. 1986. Effects of differing purified cellulose pectin, and hemicellulose fibre on faecal enzymes in 1,2-dimethyl - hydrazine-induced rat colon carcinogenesis. Cancer Research 46: 5529 - 5532)

and urease according to the instructions of the manufacturer (Boehringer Mannheim cat. no. 542946).

[0070] During the test periods, there was a decrease in the contents of β -glucuronidase, urease and β -glucosidase during the probiotic intake and the synbiotic intake (Table 8). After the intake, the enzyme levels returned to normal. The synbiotic had a stronger decreasing effect on the enzyme levels than the probiotic mixture.

Table 8. Changes in enzyme contents (nmol/min/g of feces)

	Urease		Glucuronidase		Glucosidase	
		Change%		Change%		Change%
Initial level	1080		292		746	
Probiotic	895	-17	214	-27	673	-9,8
Synbiotic	592	-45	186	-36	448	-40
Wash-Out	980		227		640	

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[0071] Metabolism of glucosidase and glucuronidase produces carcinogenic compounds. The significant decrease in enzyme activity produced by the probiotic and synbiotic combinations of the invention clearly demonstrate a positive effect with regard to the decreasing of the formation of carcinogenic substances.

15

5.3 Enterolactone content

[0072] Enterolactone content was determined using the method of Adlercreutz et al. (Adlercreutz, H., Fostis, T., Lampe, J., Wähälä, K., Mäkelä, T., Brunow, G. and Hase, T. 1993. Quantitative determination of lignans and isoflavonoids in plasma of omnivorous and vegetarian women by isotope dilution gas-chromatography mass-spectrometry. Scan J. Clin Lab Invest 53: 5 - 18.)

[0073] The enterolactone levels of test persons with an initial enterolactone level of <10 nmol/l increased significantly as a result of the synbiotic intake (to a level of 11.2).

25

[0074] No changes were observed during the test in the enterolactone levels of persons whose serum enterolactone level was normal ($10 < x < 30$) already at the beginning of the test. The results are shown in Table 9.

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Table 9. Enteronelactone contents during the test periods (grouping on the basis of initial level)

	10<x<30 nmol/l	<10 nmol/l
Initial level	24.4	3.2
Probiotic	19.8	2.6
Synbiotic	23.9	11.2

- 5 [0075] Enterolactone content has been shown to clearly correlate with the risk of getting cancer: the higher the content, the lower the risk. This result thus also shows the beneficial effect of the probiotic and synbiotic combinations of the invention to a decreased cancer risk.

5.4. Immunological studies

10 Effects on lymphocyte function

[0076] Lymphocyte function was studied before the intake of the synbiotic product was started and after 4 weeks after the intake had been started.

[0077] The lymphocyte function was studied as follows:

- 15 [0078] Lymphocytes were isolated from peripheral blood using Ficoll gradient. The lymphocytes were stimulated with PHA mitogen (Sigma) in RPMI cultivation broth (National Public Health Institute; department of nutrient broths) which contained 5% of inactivated AB+ serum (Finnish Red Cross) and L-glutamine. After 48 hours, cell culture medium was collected for cytokine determination from four adjacent culture wells having a cell density of 200 000 cells per 200 µl of culture broth in the well, either with or without the mitogen. The cells were harvested after 16 hours from the adding of thymidine and the incorporation into DNA (cpm) of radioactive thymidine was measured. The contents of cytokines IL-4, IL-5, TGF-β1 and IFN-γ were determined from the cell culture broths by using the ELISA method.

- 20 [0079] During the follow-up, no changes were detected in the IL-4, IL-5 and TGF-β1 contents secreted by the lymphocytes. The IFN-γ content secreted by PHA-stimulated lymphocytes increased significantly during the follow-up (p=0.009, Wilcoxon test, see Figure 1). Both spontaneous and PHA-stimulated proliferation of lymphocytes increased during the follow-up (p=0.0002 in both cases, Wilcoxon test, Figures 1 and 2).
- 30

[0080] According to the study results, the use of the synbiotic product thus enhances the proliferation of the lymphocytes and the secretion of IFN- γ cytokine of the test persons. IFN- γ belongs to what are known as Th1 cytokines, which strengthen cytotoxic lymphocyte function and are antagonists of IL-4 and TGF- β 1 cytokines. Low IFN- γ secretion has been reported for persons prone to allergies. In addition, children with a predisposition to atopy and allergy reactions have been presumed to suffer from slow maturing of IFN- γ secretion. The significant stimulating effect of the combinations of the invention on IFN- γ secretion thus proves their efficiency in the prevention and treatment of allergies.

CLAIMS

1. A combination **characterized** in that it comprises at least three of the microorganisms *Lactobacillus rhamnosus* LGG (ATCC 53103), *Lactobacillus rhamnosus* LC705 (DSM 7061), *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM7067) and a bifidobacterium, or *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM7067) and a bifidobacterium.
2. A combination according to claim 1, **characterized** in that it comprises *Lactobacillus rhamnosus* LGG (ATCC 53103), *Lactobacillus rhamnosus* LC705 (DSM 7061), *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM7067) and a bifidobacterium.
3. A combination according to claim 1, **characterized** in that it comprises *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM7067) and a bifidobacterium.
4. A combination according to any one of claims 1 to 3, **characterized** in that the bifidobacterium is *Bifidobacterium infantis* Bbi99 (DSM 13692).
5. A combination according to any one of claims 1 to 4, **characterized** in that it further comprises conventional starter microbes.
6. A combination according to any one of claims 1 to 5, **characterized** in that it also comprises a prebiotic or that it is used together with a prebiotic.
7. A combination according to claim 6, **characterized** in that the prebiotic is an oligosaccharide, xylo-oligomer or polydextrose.
8. A combination according to claim 7, **characterized** in that the prebiotic is a galacto-oligosaccharide (GOS).
9. A combination according to any one of claims 1 to 8 to be used as a therapeutic substance.
10. A combination comprising at least two of the following microorganisms: *Lactobacillus rhamnosus* LGG (ATCC 53103), *Lactobacillus rhamnosus* LC705 (DSM 7061), *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM7067) and a bifidobacterium, although not the combination *Lactobacillus rhamnosus* LGG (ATCC 53103) and a bifidobacterium, to be used as a therapeutic substance.

11. Use of a combination according to any one of claims 1 to 8 in food industry, pharmaceutical industry, or in the manufacture of health-promoting products or natural products.

12. Use according to claim 11, **characterized** in that the combination is added to a dairy product, drink, juice, soup or children's food.

13. Use according to claim 11, **characterized** in that the combination is prepared in a single dose form.

14. Use according to claim 13, **characterized** in that the dose form is a capsule.

FIGURE 1.

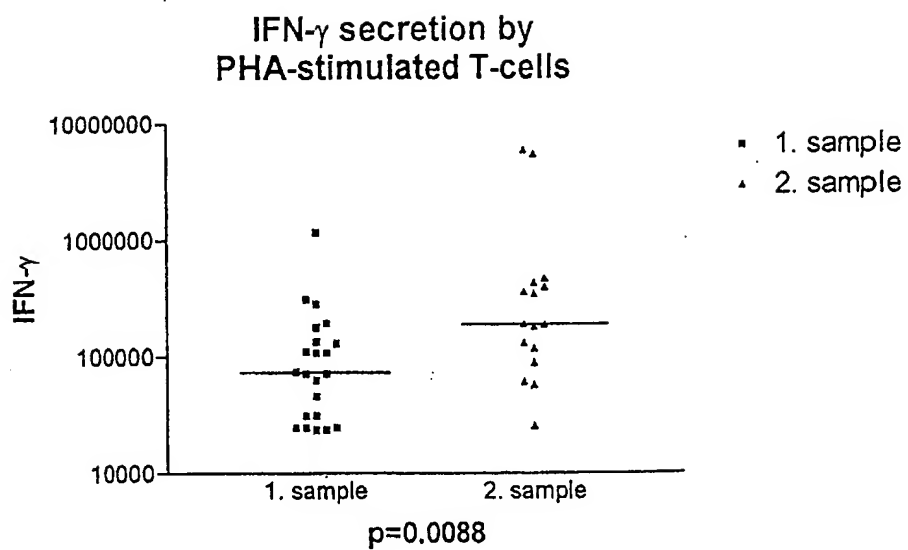
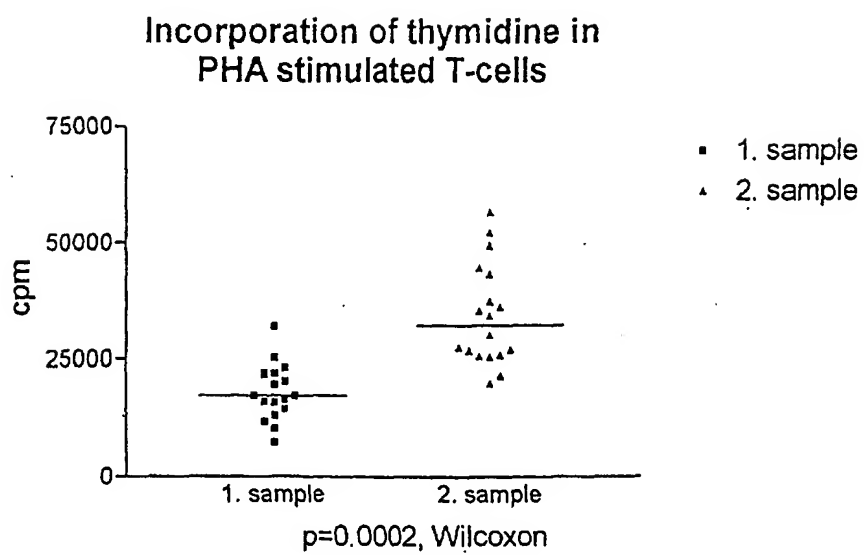


FIGURE 2.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 02/00035

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A23L 1/03, A61K 35/74

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A23L, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Lait, volume 79, 1999, Tarja H. Suomalainen et al: "Propionic acid bacteria as protective cultures in fermented milks and breads", page 165 - page 174, see abstract --	1-14
X	Clinical and Diagnostc Laboratory Immunology, Volume 6, No. 6, November 1999, Pirkka V. Kirjavainen et al: "Effects of Orally Administered Viable Lactobacillus rhamnosus GG and Propionibacterium freudenreichii subsp. shermanii JS on Mouse Lymphocyte Proliferation", page 799 - page 802, see abstract --	1-14

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

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"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Name and mailing address of the ISA/

Swedish Patent Office

Box 5055, S-102 42 STOCKHOLM

Facsimile No. +46 8 666 02 86

Authorized officer

Yvonne Siösteen/EÖ

Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Milchwissenschaft, Volume 53, No. 11, 1998, S. Sarkar et al: "Process for the manufacture of a new modified cultured milk product for infants and children", page 603 - page 605 --	1-14
A	US 5378458 A (ANNIKA MÄYRÄ-MÄKINEN ET AL), 3 January 1995 (03.01.95) --	1-14
A	WO 0071138 A2 (REID, GREGOR), 30 November 2000 (30.11.00) --	1-14
A	EP 1020123 A1 (SITIA-YOMO S.P.A.), 19 July 2000 (19.07.00) --	1-14
A	WO 9734615 A1 (THE UNIVERSITY OF NEW SOUTH WALES), 25 Sept 1997 (25.09.97) -- -----	6-8

INTERNATIONAL SEARCH REPORT
Information on patent family members

28/01/02

International application No.
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(71) Applicant (for all designated States except US): VALIO LTD [FI/FI]; Meijeritie 6, FIN-00370 Helsinki (FI).

(72) Inventors; and

(75) Inventors/Applicants (for US only): MÄYRÄ-MÄKINEN, Annika [FI/FI]; Maurinkatu 4 B 19, FIN-00170 Helsinki (FI). SUOMALAINEN, Tarja [FI/FI]; Valkealantie 10 A 1, FIN-00950 Helsinki (FI). VAARALA, Outi [FI/FI]; Fredrikinkatu 75 A 5, FIN-00100 Helsinki (FI).

(74) Agent: KOLSTER OY AB; Iso Roobertinkatu 23, P.O. Box 148, FIN-00121 Helsinki (FI).

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Declarations under Rule 4.17:

— as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)

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(54) Title: COMBINATION OF PROBIOTICS

(57) Abstract: The invention relates to a probiotic combination comprising different combinations of lactobacilli, propionic acid bacteria and/or bifidobacteria. The probiotics are preferably combined with a suitable prebiotic to form a synbiotic. The combination of the invention can be consumed as such or combined with a suitable foodstuff or pharmaceutical product, and it is therapeutically useful for example for stimulating the immune system and for general health improvement.

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